Diaphorase catalyzed biotransformation of RDX via N-denitration mechanism

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Abstract

Previously, we hypothesized that hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) can be biotransformed by anaerobic sludge via three different routes: (1) direct ring cleavage via α-hydroxylation of a-CH_2 group, (2) reduction of one of the –NO_2 groups to –NO, (3) N-denitration prior to ring cleavage. The present study describes biotransformation of RDX via route 3 by a diaphorase (EC 1.8.1.4) from *Clostridium kluyveri* using NADH as electron donor. The removal of RDX was accompanied by the formation and accumulation of nitrite ion (NO_2^-), formaldehyde (HCHO), ammonium (NH_4^+), and nitrous oxide (N_2O). None of the RDX-nitroso products were detected. The ring cleavage product methylenedinitramine was detected as the transient intermediate. Product stoichiometry showed that each reacted RDX molecule produced one nitrite ion and the product distribution gave a carbon (C) and nitrogen (N) mass balance of 91 and 92%, respectively, supporting the occurrence of a mono-denitration step prior to the ring cleavage and decomposition. Severe oxygen mediated inhibition (92% inhibition) of RDX biotransformation and superoxide dismutase-sensitive cytochrome c reduction indicated the potential involvement of an anion radical RDX$^{\cdot-}$ prior to denitration. A comparative study between native- and apo-enzymes showed the possible involvement of flavin mononucleotide (FMN) in catalyzing the transfer of a redox equivalent (e/H^+) from NADH to RDX to produce RDX$^{\cdot-}$ responsible for secondary decomposition. © 2002 Elsevier Science (USA). All rights reserved.

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Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a cyclic nitramine explosive commonly used for military and commercial purposes. The manufacturing, use, and disposal of RDX have resulted in severe soil and ground water contamination [1,2]. RDX is toxic, mutagenic and carcinogenic to humans, and other biological systems [3,4], hence there is an urgent need for its safe removal from the environment.

Recently, we reported that RDX can be easily degraded with anaerobic sludge to produce HCHO, CO_2, N_2O, and NH_3 [5,6], but we did not elaborate on the enzymes responsible for initiating RDX degradation. Kitts et al. [7] have reported the reduction of RDX by a type I (two-electron transfer process) oxygen-insensitive nitroreductase without providing details on either products or degradation pathways. Whereas Shah and

Spain [8] reported N-denitrohydrogenation of tetryl by a ferredoxin–NADP oxidoreductase (EC 1.18.1.2) using NADPH as electron donor. In a more recent study, we found that RDX can be transformed by a nitrate reductase from *Aspergillus niger* to produce hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) via a two-electron transfer process prior to its decomposition to HCHO and N_2O [9].

For the present study, we selected a flavoenzyme, diaphorase, from an anaerobic bacterium *Clostridium kluyveri* to biotransform RDX. *Clostridium* species are common inhabitants of anaerobic environments and thus we expect diaphorase to be one of the key enzyme(s) responsible for RDX degradation by the anaerobic sludge [5,6]. Previously, *Clostridium* species have been known to biotransform RDX and nitroaromatic explosive, 2,4,6-trinitrotoluene (TNT) [10]. A carbon monoxide dehydrogenase from *Clostridium thermoaceticum* reportedly biotransformed TNT [11]. However, no

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detailed study has been reported so far in regard to biotransformation of RDX by a purified enzyme from *Clostridium* species.

The diaphorase (EC 1.8.1.4) from *C. kluyveri* is a 24 kDa flavoenzyme, containing 1 mol of FMN per mole of enzyme, and it catalyzes the pyridine nucleotide-dependent reduction of dyes [12]. This enzyme catalyzed an oxygen-sensitive one-electron reduction of nitrofluorenes [13]. Our objective in the present study was to identify the metabolites and to understand the mechanism of RDX biotransformation catalyzed by the a diaphorase (EC 1.8.1.4) from *C. kluyveri*. The knowledge of the initial steps involved in enzymatic degradation of RDX would improve our understanding of the degradation process, which might lead to the optimized mineralization of this energetic chemical under field conditions.

Materials and methods

Chemicals. Commercial grade RDX (purity > 99%) was provided by Defense Research and Development, Que., Canada. NADH, flavin mononucleotide (FMN), 2,6-dichlorophenol-indophenol (DPIP), superoxide dismutase (SOD, EC 1.15.1.1, from bovine erythrocytes), cytochrome c (from horse heart, MW 12,384 Da), and formaldehyde were purchased from Sigma Chemicals, Canada. Methylenedinitramine was obtained from the rare chemical department of Aldrich, Canada. Standard nitrous oxide (N_2O, 980 ppm by mol) was obtained from Scott specialty gases, Sarnia, ON, Canada. All other chemicals were of highest purity grade available.

Enzyme preparation. Diaphorase (EC 1.8.1.4) from *C. kluyveri* was obtained from Sigma Chemicals, Canada, as a lyophilized powder. The enzyme was suspended in 50 mM potassium phosphate buffer (pH 7.0) and filtered through a Biomax-5K membrane (Sigma Chemicals) before resuspension in the same buffer. The protein concentration was measured by Bicinchoninic Acid (BCA) Kit (Sigma Chemicals) using bovine serum albumin as standard. The native enzyme activity was estimated (as per company guidelines) spectrophotometrically at 340 nm as the rate of oxidation of NADH using 2,6-dichlorophenol-indophenol as the electron acceptor.

Biotransformation assays. RDX biotransformation assays with diaphorase were performed in 6 ml glass vials under anaerobic conditions (with an atmosphere of argon) at pH 7.0 and 27 °C. Each vial contained RDX (100 µM), NADH (150 µM), and 50 µl of the enzyme (0.5 native units) in a final volume of 1 ml potassium phosphate buffer (50 mM, pH 7.0). Three different controls were prepared. The first control contained RDX, NADH, and buffer without enzyme; the second contained RDX, enzyme, and buffer without NADH, and the third contained only RDX and buffer. The reaction time was 1 h unless stated otherwise. Samples from the liquid and gas phases in the vials were withdrawn periodically to analyze for RDX and the transformed products as described below. RDX transformation activity of enzyme was expressed as µmol of RDX transformed h^{-1} mg^{-1} protein.

Inhibition with oxygen. The effect of oxygen on RDX transformation activity of diaphorase was studied by performing the assays under aerobic and anaerobic conditions at pH 7.0 and 27 °C. In another experiment, we determined the role of O_2 transformation to superoxide radical anion ($O^{\cdot-}$) in the inhibition of RDX biotransformation. This was done by incubating the RDX with diaphorase in the presence of NADH, cytochrome c (100 µM), and SOD (150 µg/ml) as described previously [14]. The absorption of reduced cytochrome c was monitored at 550 nm.

Preparation of apoenzyme (deflavo enzyme) and its reconstitution. The deflavo form of diaphorase was prepared as described before [15,16] with some modifications. The holoenzyme was dialyzed for 48 h at 4 °C against a dialysis solution composed of 100 mM potassium phosphate buffer (pH 7.0), EDTA (0.1 mM), glycerol (10% v/v), and KBr (3 M). The dialysis buffer was changed every 6 h. The reconstitution of apoenzyme was carried out in ice cold potassium phosphate buffer (pH 7.0) in the presence of glycerol (10% v/v). FMN was added at variable concentrations (0–250 µM) to the apoenzyme preparation. The unbound FMN was removed by washing the enzyme with the same buffer using Biomax-5K membrane centrifuge filter units. The enzyme activity was assayed after each addition of FMN to the apoenzyme to determine the concentration-dependent reconstitution of apoenzyme by the FMN.

Analytical procedures. RDX was analyzed by a reversed phase-high performance liquid chromatograph (RP-HPLC) connected to photodiode array (PDA) detector ($\lambda_{254\,nm}$). Samples (50 µl) were injected into a Supelcosil LC-CN column (4.6 mm ID × 25 cm) (Supelco, Bellafonte, PA) and the analytes were eluted using methanol/water gradient at a flow rate of 1.5 ml/min [5]. Methylenedinitramine was analyzed by a Micromass bench-top single quadrupole mass detector attached to a Hewlett-Packard 1100 series HPLC system equipped with a PDA detector. Ionization was carried out in a negative electrospray ionization mode ES(–) producing mainly the deprotonated molecular mass ions [M–H]. Methylenedinitramine was detected as its deprotonated molecular mass ion [M–H] at 135 Da and confirmed by comparison with a reference standard [6]. Nitroso-derivatives of RDX (i.e., hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine, MNX; hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine, DNX, and hexahydro-1,3,5-trinitroso-1,3,5-triazine, TNX), nitrite (NO_2^-), ammonium (NH_4^+), formaldehyde (HCHO), and nitrous oxide (N_2O) were analyzed as reported [5,6].
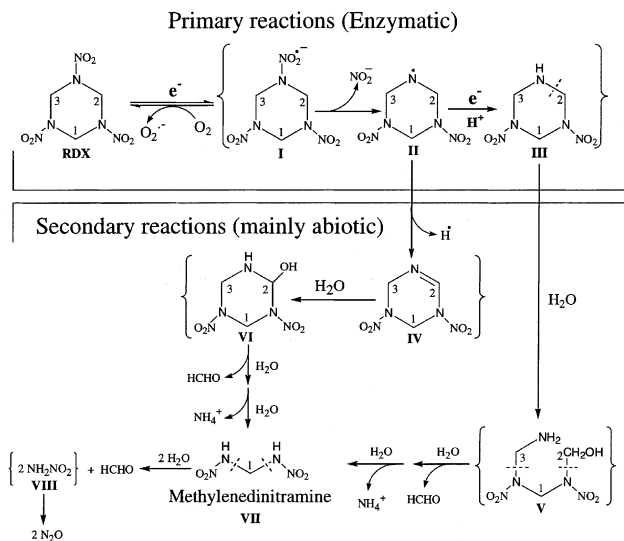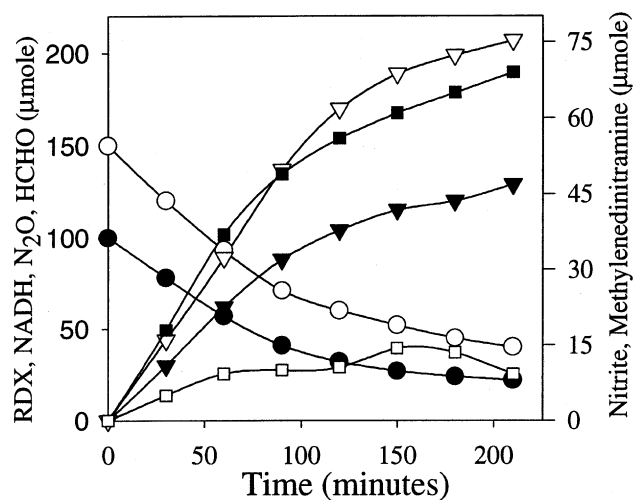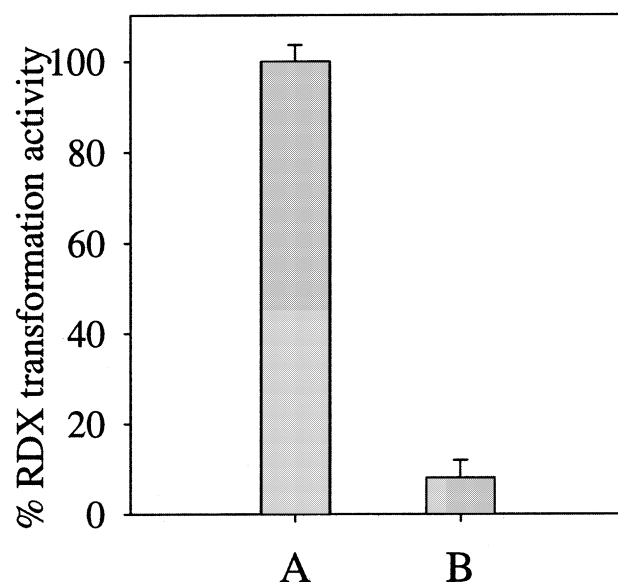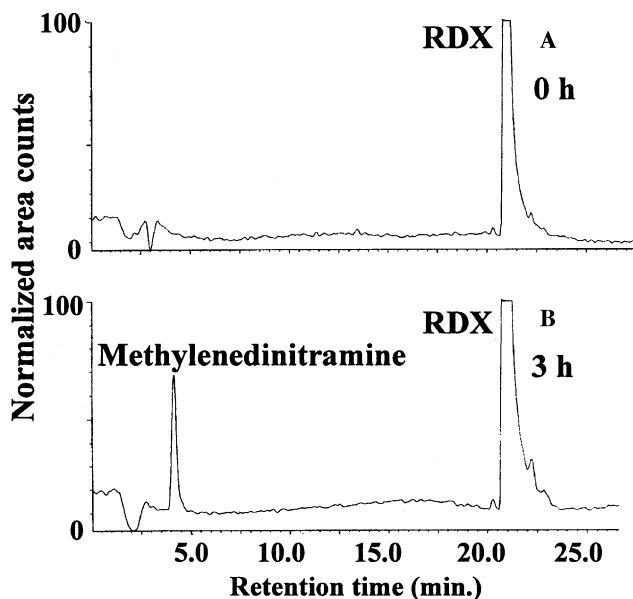
Results and discussion

RDX biotransformation and product identification

Clostridium kluyveri diaphorase was found to transform RDX at pH 7.0 and 27 °C under anaerobic conditions using NADH as the electron donor. A typical LC/MS chromatogram of RDX transformation with diaphorase showed the production of methylenedinitramine as a key ring cleavage metabolite at a retention time of 4.2 min as shown in Fig. 1. None of the nitroso-RDX intermediates, particularly MNX, were detected, although such initial RDX reduced products were frequently observed during RDX degradation by anaerobic sludge [5,6]. No biotransformation of RDX was observed in the control experiments that did not contain enzyme and/or NADH. The time course of the reaction showed that the disappearance of RDX and NADH was accompanied by the formation and accumulation of HCHO, N_2O, and NO_2^- (Fig. 2).

Inhibition by oxygen

We found that the presence of oxygen (O_2) inhibited (92%) the transformation of RDX as shown in Fig. 3, clearly supporting the involvement of an oxygen-sensitive step during the initial enzymatic attack on RDX. We observed a negligible oxidation of NADH under

Fig. 1. LC/MS (ES-) chromatogram of RDX and diaphorase reaction showing methylenedinitramine as a key RDX intermediate. (A) 0 h reaction; (B) 3 h reaction.

Fig. 2. Time course of diaphorase catalyzed RDX transformation with simultaneous production of intermediate and end-products. Symbols: (●) RDX; (○) NADH; (▼) N_2O; (▽) HCHO; (■) nitrite; (□) methylenedinitramine. Standard deviations were within 6% of the absolute mean values ($n = 3$).

Fig. 3. Effect of oxygen on diaphorase catalyzed RDX transformation. (A) Anaerobic conditions; (B) aerobic conditions. Hundred percentage RDX transformation activity was equivalent to 47 μmol RDX transformed h^{-1} mg^{-1} protein.

Fig. 4. Proposed pathway of diaphorase catalyzed RDX transformation based on stoichiometry of metabolites recovered and NADH consumed. Primary reactions involve RDX reduction to RDX anion radical I that undergoes denitrohydrogenation to produce III. Secondary reactions involve ring cleavage and spontaneous decomposition in water. Intermediates shown inside brackets were not detected.

aerobic conditions within the assay time of 1 h, indicating that O_2 inhibition of RDX biotransformation was not due to the depletion of NADH. In another experiment, we observed that diaphorase did not catalyze the electron transfer from NADH to O_2 and this observation was similar to the one reported by Kaplan et al. [12] using the same enzyme [12]. Based on these observations, we hypothesized that inhibition of RDX transformation by O_2 is most probably due to quenching of an unpaired electron from the RDX anion radical

$(RDX^{\cdot-})$ by O_2. Such reaction would revert $RDX^{\cdot-}$, an initial transformation product, to its original structure RDX producing instead $O^{\cdot-}$ thus preventing the molecule from further decomposition (Fig. 4) as was the case with tetryl [8,14]. $RDX^{\cdot-}$ appears to be formed via an initial electron transfer process catalyzed by diaphorase (Fig. 4). Ritter and Malejka-Giganti [13] also reported the reduction of a nitro group in nitrofluorene

compounds via an O_2-sensitive one-electron transfer process catalyzed by a diaphorase from *C. kluyveri*.

To test the hypothesis that O_2 inhibited the transformation of RDX by quenching an electron from RDX$^{\cdot-}$, we incubated RDX with diaphorase in the presence of NADH, SOD and cytochrome c. It was observed that SOD inhibited the reduction of cytochrome c by 37% thus providing evidence for the potential involvement of redox cycling between RDX$^{\cdot-}$ and RDX through which O_2 was converted to $O^{\cdot-}$ (Fig. 4). Control experiments without diaphorase showed that RDX can neither be auto-oxidized nor it can reduce cytochrome c.

C- and N-mass-balance and stoichiometry of the reaction

The reaction stoichiometry (Table 1) indicated that one nitrite molecule was produced per reacted RDX molecule. On the other hand, 78 μmol of the reacted RDX produced 129 μmol N_2O and 9.2 μmol of methylenedinitramine. The latter is unstable in water at pH 7.0 and 27 °C and decomposes quantitatively to produce stoichiometric amounts of N_2O (two molecules) and HCHO (one molecule) [6]. Considering that all of the N_2O produced during the reaction comes from the decomposition of methylenedinitramine, it was concluded that one molecule of methylenedinitramine was produced for each reacted RDX molecule. The total nitrogen mass recovery was 91% and was distributed as N_2O (55%), nitrite (15%), ammonium (13%), and methylenedinitramine (8%). The N-mass-balance data revealed that of the six nitrogen atoms in one RDX molecule, four atoms were finally recovered as nitrous oxide (two N_2O molecules) whereas the fifth and sixth atoms were present in nitrite (NO_2^-) and ammonium (NH_4^+), respectively, (Table 1).

The total carbon mass recovery was 92% and it was distributed as formaldehyde (88%) and methylenedi-

nitramine (4%). The latter, as mentioned above, decomposes quantitatively to HCHO and N_2O, indicating that all the three carbon atoms in RDX were finally recovered in the form of HCHO.

Numerically, 1.4 μmol of NADH was consumed to biotransform 1.0 μmol of RDX (Table 1). The stoichiometry of NADH consumption vs RDX transformation supports that one molecule of NADH ($2e^-/2H^+$) was utilized per RDX molecule for its initial denitration. The remaining 0.4 μmol was either utilized by other transient intermediates/end-products following the ring cleavage or bound to protein and thus prevented their detection. The other possibility is that some NADH may be consumed during the futile redox cycling in the presence of traces of oxygen in the reaction medium.

Proposed mechanism of biotransformation

Based on the oxygen sensitivity, product identification, C- and N-mass-balance, and stoichiometry of the reaction, we propose that RDX undergoes a mono-denitration process which was responsible and sufficient for the ring cleavage and secondary decomposition. The absence of nitroso products such as MNX, DNX, and/or TNX also supported the denitration of RDX as the main reaction step responsible for the ring cleavage.

The oxygen inhibition (92%) of RDX transformation and SOD-sensitive cytochrome c reduction experiments suggested that the transfer of a net two redox equivalents ($2e^-/H^+$) to the RDX, as determined by stoichiometry of NADH, occurred in a stepwise manner. First step produce RDX$^{\cdot-}$ (I) whose spontaneous denitration would generate nitrite and the free radical RDX$^{\cdot}$ (II) (Fig. 4). In the second step, RDX$^{\cdot}$ (II) either undergoes H-abstraction to form the amine derivative (III) by acquiring a second redox equivalent (e^-/H^+) or can lose a hydrogen atom to form the cyclohexene derivative (IV) (Fig. 4). However, the stoichiometry of NADH con-

Table 1
Carbon and nitrogen mass balance and stoichiometry of reactants consumed and metabolites produced during RDX transformation catalyzed by a diaphorase from *Clostridium kluyveri* at pH 7.0 and 27 °C

Reactants/metabolites	Amount of reactants/metabolites (μmol)	% Carbon recovery[a]	% Nitrogen recovery[a]
Reactants consumed			
RDX	78.0	100.0	100.0
NADH	110.0	NA	NA
Metabolites produced			
Methylenedinitramine	9.2	4.0	8.0
Formaldehyde (HCHO)	207.0	88.0	NA
Nitrite (NO_2^-)	69.0	NA	15.0
Nitrous oxide (N_2O)	129.0	NA	55.0
Ammonium (NH_4^+)	62.0	NA	13.0
Total % mass recovery		92.0	91.0

[a] Calculated from the total carbon and nitrogen mass in the transformed RDX (78 μmol). Initial RDX and NADH concentrations were 100 and 150 μmol, respectively. NA: not applicable. The standard deviations were within 6% of the absolute mean values ($n = 3$).

sumption favors the formation of amine derivative (III). The formation of amine derivative (III) via a H-abstraction by the RDX· (II) during photolysis of RDX has been reported [17] previously, but to the best of our knowledge no similar biological reactions are known. However, Anusevicius et al. [14] reported two single e-transfer steps during N-denitration of tetryl catalyzed by a mammalian DT-diaphorase (EC 1.6.99.2).

The hypothetical intermediates III and IV might be unstable in water and therefore underwent hydrolytic ring cleavage. As a result, III would produce V, and IV would give rise to VI. Further, the hypothetical intermediates V and VI decompose in water and stoichiometrically produce methylenedinitramine (VII), HCHO and NH_4^+ (Fig. 4). Methylenedinitramine, being unstable in water also decomposes to produce HCHO and N_2O [6]. Although, we detected methylenedinitramine quantitatively but we were unable to detect its hypothetical precursor intermediates V and VI.

The proposed pathway in Fig. 4 is consistent with the experimental mass balance of carbon and nitrogen (Table 1). Chapman et al. [18] reported a similar, but non-enzymatic N-denitrohydrogenation of HMX (a cyclicnitramine explosive) using 1-benzyl-1,4-dihydronicotinamide instead of NADH, however, this reaction needed an initiation by sodium dithionite or light. On the other hand, N-denitrohydrogenation of tetryl using a ferredoxin-NADP+ oxidoreductase (EC 1.18.1.2) [8] and a mammalian DT-diaphorase (EC 1.6.99.2) [14] has also been reported.

Possible involvement of FMN in RDX transformation

FMN is a redox cofactor of *C. kluyveri* diaphorase and it is present in the ratio of 1 mol/mol of enzyme [12]. In the present study, the deflavo enzyme (apoenzyme) lost 87.5% of the RDX transformation activity (Fig. 5), suggesting that FMN is an active redox center that possibly mediates the transfer of electrons from NADH to RDX. The remaining 12.5% enzymatic activity may be due to the incomplete removal of FMN from the native enzyme. We found that when the deflavo enzyme was reconstituted with increasing concentration of FMN, there was a gradual increase in RDX transformation activity up to a maximum of 85% at the FMN concentration of 200 μM (Fig. 6). In another experiment, we found that a commercially available FMN in free form can transform RDX using NADH as electron donor. However, the transformation rate of the free FMN was comparatively lower than the diaphorase-bound FMN (Fig. 6). These results indicate that FMN plays a crucial role in the reduction of RDX by acting as a possible site of electron transfer reaction. In this context, Khan et al. [19], showed that reduced FMN in pentaerythritol tetranitrate reductase caused the reduction of nitroester explosives (glycerol trinitrate and pentaerythritol tetranitrate) and nitroaromatic explosives (TNT and picric acid). Other reports also showed that the flavin moieties (FAD and FMN) in the flavoenzymes play a key role in substrate reduction [15,20,21].

In conclusion, the present study showed that the diaphorase catalyzed N-denitration of RDX is an oxygen-sensitive reaction which possibly requires a net two redox equivalents. Based on the present experimental data and its analogy with other reported systems [8,13,14] we proposed that the first redox equivalent leads to the transformation of RDX to RDX·⁻ whose subsequent denitration leads to RDX·. The second redox equivalent possibly causes hydrogenation of RDX· leading to the
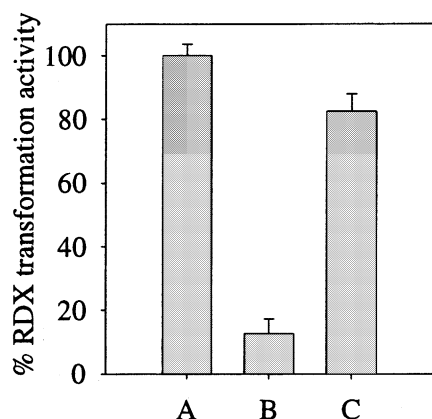

Fig. 5. Role of FMN in RDX transformation activity of diaphorase. (A) Native enzyme; (B) apoenzyme (deflavo form); (C) apoenzyme reconstituted with 200 μM FMN. Hundred percentage activity was equivalent to 41 μmol RDX transformed h^{-1} mg^{-1} protein.
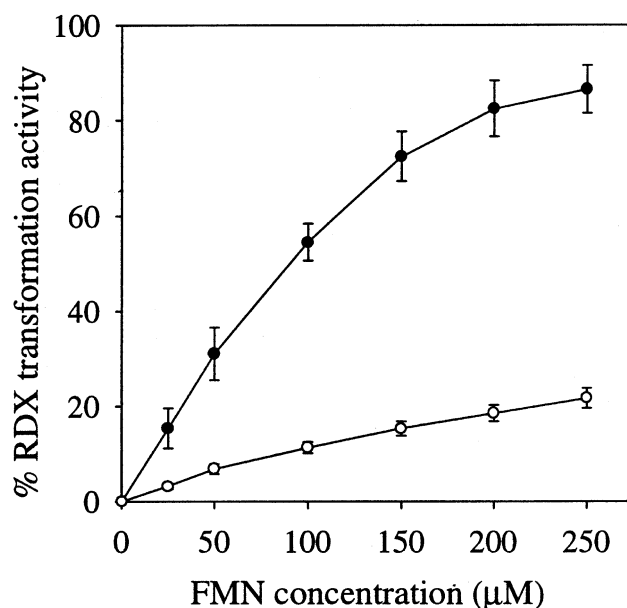

Fig. 6. Concentration-dependent reconstitution of diaphorase by FMN. Symbols: (•) percentage restored activity of diaphorase; (○) FMN (commercially available) catalyzed RDX transformation. Hundred percentage activity was equivalent to 41 μmol RDX transformed h^{-1} mg^{-1} protein.

formation of corresponding amine (III) (Fig. 4). The latter undergoes spontaneous hydrolytic decomposition in water to produce the transient intermediates and end-products. FMN seems to play a key role in transferring the redox equivalents from NADH (donor) to RDX (acceptor).

One of the significance of the present study is that *C. kluyveri* diaphorase catalyzed the RDX transformation at the expense of a net two redox equivalents per RDX molecule which is apparently different and more economical than the earlier reported conventional RDX biotransformation route, i.e., via MNX formation [5–7]. In a recent study, the MNX route of RDX transformation utilized a net six electrons to transform one RDX molecule before its ring cleavage and decomposition [9]. This study also revealed that the transformation of at least one nitro group of RDX is necessary and sufficient to initiate the ring cleavage and subsequent decomposition. The present study is unique of its kind since enzymology of cyclicnitramine compounds such as RDX are not well known and hence more work is needed in this area to understand and optimize the bioremediation process of these compounds.

References

[1] R. Haas, E.v.L. Schreiber, G. Stork, Conception for the investigation of contaminated munitions plants. 2. Investigation of former RDX-plants and filling stations, Fresenius J. Anal. Chem. 338 (1990) 41–45.

[2] C.A. Myler, W. Sisk, in: G.S. Sayler, R. Fox, J.W. Blackburn (Eds.), Environmental Biotechnology for Waste Treatment: Bioremediation of Explosives Contaminated Soils (Scientific Questions/Engineering Realities), Plenum Press, New York, 1991, pp. 137–146.

[3] J. Yinon, Toxicity and metabolism of explosives, CRC Press, Boca Raton, FL, 1990.

[4] S.S. Talmage, D.M. Opresko, C.J. Maxwel, C.J.E. Welsh, F.M. Cretella, P.H. Reno, F.B. Daniel, Nitroaromatic munition compounds: environmental effects and screening values, Rev. Environ. Contam. Toxicol. 161 (1999) 1–156.

[5] J.A. Hawari, A. Halasz, S. Beaudet, C. Groom, L. Paquet, C. Rhofir, G. Ampleman, S. Thiboutot, Characterization of metabolites during biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) with municipal anaerobic sludge, Appl. Environ. Microbiol. 66 (2000) 2652–2657.

[6] A. Halasz, J. Spain, L. Paquet, C. Beaulieu, J. Hawari, Insights into the formation and degradation mechanisms of methylenedinitramine during the incubation of RDX with anaerobic sludge, Environ. Sci. Technol. 36 (2002) 633–638.

[7] C.L. Kitts, C.E. Green, R.A. Otley, M.A. Alvarez, P.J. Unkefer, Type I nitroreductases in soil enterobacteria reduce TNT (2,4,6-trinitrotoluene) and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine), Can. J. Microbiol. 46 (2000) 278–282.

[8] M.M. Shah, J.C. Spain, Elimination of nitrite from the explosive 2,4,6-trinitrophenylmethylnitramine (tetryl) catalyzed by ferredoxin NADP oxidoreductase from spinach, Biochem. Biophys. Res. Commun. 220 (1996) 563–568.

[9] B. Bhushan, A. Halasz, J. Spain, S. Thiboutot, G. Ampleman, J. Hawari, Biotransformation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) catalyzed by a NAD(P)H: nitrate oxidoreductase from *Aspergillus niger*, Environ. Sci. Technol. 36 (2002) 3104–3108.

[10] K.M. Regan, R.L. Crawford, Characterization of *Clostridium bifermentans* and its biotransformation of 2,4,6-trinitrotoluene (TNT) and 1,3,5-triaza-1,3,5-trinitro cyclohexane (RDX), Biotechnol. Lett. 16 (1994) 1081–1086.

[11] S. Huang, P.A. Lindahl, C. Wang, G.N. Bennett, F.B. Rudolph, J.B. Hughes, 2,4,6-Trinitrotoluenreduction reduction by carbon monoxide dehydrogenase from *Clostridium thermoaceticum*, Appl. Environ. Microbiol. 66 (2000) 1474–1478.

[12] F. Kaplan, P. Setlow, N.O. Kaplan, Purification and properties of a DPNH–TPNH diaphorase from *Clostridium kluyveri*, Arch. Biochem. Biophys. 132 (1969) 91–98.

[13] C.L. Ritter, D. Malejka-Giganti, Nitroreduction of nitrated and C-9 oxidized fluorenes in vitro, Chem. Res. Toxicol. 11 (1998) 1361–1367.

[14] Z. Anusevicius, J. Sarlauskas, H. Nivinskas, J. Segura-Aguilar, N. Cenas, DT-diaphorase catalyzes N-denitration and redox cycling of tetryl, FEBS Lett. 436 (1998) 144–148.

[15] R.A. Madigan, S.G. Mayhew, Preparation of the apoenzyme of the FMN-dependent *Clostridium kluyveri* diaphorase by extraction with apoflavodoxin, Biochem. Soc. Trans. 22 (1993) 57S.

[16] G. Tedeschi, S. Chen, V. Massey, Active site studies of DT-diaphorase employing artificial flavins, J. Biol. Chem. 270 (1995) 2512–2516.

[17] G.R. Peyton, M.H. LeFaivre, S.W. Maloney, in: Verification of RDX photolysis mechanism, CERL Technical Report 99/93, 40161102B25, Environmental Research, Engineer Research and Development Center, Champaign, IL, 1999.

[18] R.D. Chapman, R.A. O'Brien, P.A. Kondracki, N-denitration of nitramines by dihydronicotinamides, Tetrahedron 52 (1996) 9655–9664.

[19] H. Khan, R.J. Harris, T. Barna, D.H. Craig, N.C. Bruce, A.W. Munro, P.C.E. Moody, N.S. Scrutton, Pentaerythritol tetranitrate reductase: kinetic and structural basis of reactivity with NADPH, 2-cyclohexenone, nitroesters and nitroaromatic explosives, J. Biol. Chem. 277 (2002) 21906–21912.

[20] H. Lindsay, E. Beaumont, S.D. Richards, S.M. Kelly, S.J. Sanderson, N.C. Price, J.G. Lindsay, FAD insertion is essential for attaining the assembly competence of the dihydrolipoamide dehydrogenase (E3) monomer from *Escherichia coli*, J. Biol. Chem. 275 (2000) 36665–36670.

[21] L. Xia, M. Bjornstedt, T. Nordman, L.C. Eriksson, J.M. Olsson, Reduction of ubiquinone by lipoamide dehydrogenase: an antioxidant regenerating pathway, Eur. J. Biochem. 268 (2001) 1486–1490.